New frontiers in intravital microscopy of the kidney

Hall, Andrew M ; Schuh, Claus D ; Haenni, Dominik

Abstract: Purpose of review Intravital imaging with multiphoton microscopy enables the detailed study of dynamic cellular processes within functioning organs in living animals, in ways that would not otherwise be possible. It therefore represents a powerful tool in translational kidney research. In this article, we will discuss several new technical developments that have significantly increased the capabilities of kidney imaging. Recent findings Important contemporary advances in biomedical imaging technology include longer wavelength excitation lasers, far-red emitting fluorescent reporters, highly sensitive detectors, fluorescence lifetime measurements, adaptive optics, microendoscopes, high-throughput automated analysis algorithms and tissue clearing techniques. Several recent studies have utilized intravital microscopy to gain valuable new insights into important physiological and pathophysiological processes in the kidney, such as renal handling of albumin and the cellular pathogenesis of acute kidney injury in sepsis. Summary Major technological advances are rapidly expanding the frontiers of intravital microscopy, which is likely to play an increasingly important role in preclinical kidney research in the coming years.

DOI: https://doi.org/10.1097/MNH.0000000000000313

New frontiers in intravital microscopy of the kidney

Andrew M. Hall[a,b], *Claus D. Schuh*[a], *and Dominik Haenni*[a,c]

Purpose of review
Intravital imaging with multiphoton microscopy enables the detailed study of dynamic cellular processes within functioning organs in living animals, in ways that would not otherwise be possible. It therefore represents a powerful tool in translational kidney research. In this article, we will discuss several new technical developments that have significantly increased the capabilities of kidney imaging.

Recent findings
Important contemporary advances in biomedical imaging technology include longer wavelength excitation lasers, far-red emitting fluorescent reporters, highly sensitive detectors, fluorescence lifetime measurements, adaptive optics, microendoscopes, high-throughput automated analysis algorithms and tissue clearing techniques. Several recent studies have utilized intravital microscopy to gain valuable new insights into important physiological and pathophysiological processes in the kidney, such as renal handling of albumin and the cellular pathogenesis of acute kidney injury in sepsis.

Summary
Major technological advances are rapidly expanding the frontiers of intravital microscopy, which is likely to play an increasingly important role in preclinical kidney research in the coming years.

Keywords
intravital microscopy, kidney, multiphoton imaging

INTRODUCTION

Intravital fluorescence microscopy offers the tantalizing prospect of visualizing dynamic cellular processes at high resolution within functioning organs in a living animal and how they are altered in disease states. Multiphoton microscopy (MPM) is a form of fluorescence imaging that uses a long wavelength pulsed excitation laser (typically in the range 700–1000 nm), relying on the basic principle that two or more low-energy photons arriving simultaneously can excite a fluorescent molecule [1]. It has significant advantages over conventional confocal microscopy when working with intact tissues, including increased depth of imaging and decreased phototoxicity. Intravital imaging with MPM is a powerful and rapidly expanding technique in preclinical research, which is driving a much-needed renaissance in whole animal physiology [2–5]. In this article, we will first summarize some important recent technical advances that have significantly broadened the scope of intravital microscopy and will then discuss how these are yielding important new insights into kidney function and diseases.

NEW FRONTIERS IN IMAGING TECHNOLOGY

The capabilities of biological imaging with MPM have rapidly expanded in the last few years, due to parallel advances in several distinct, but related, fields. Major new developments include (but are not limited to) longer wavelength excitation lasers, highly sensitive detectors, automated microscopes capable of running independently over days, microendoscopes, tissue clearing techniques to increase transparency, exponential increases in computational power, software algorithms that can perform high-throughput and unbiased automated analysis with large data sets, an ever expanding range of functional reporters (many of which with ratio-metric readouts for increased reliability) and increasing availability of 'off the shelf' genetically modified animals stably expressing fluorescent molecules. A separate detailed discussion of each of these advances is beyond the scope of this article, but in general they are all characteristic of the wider technological revolution that is both shaping and

[a]Institute of Anatomy, University of Zurich, [b]Department of Nephrology, University Hospital Zurich and [c]Center for Microscopy and Image Analysis, University of Zurich, Zurich, Switzerland

Correspondence to Andrew M. Hall, PhD, MRCP, Institute of Anatomy, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland. Tel: +41 044 635 52 25; fax: +41 0 44 635 57 02; e-mail: andrew.hall@uzh.ch

Curr Opin Nephrol Hypertens 2017, 26:172–178
DOI:10.1097/MNH.0000000000000313

KEY POINTS

- Multiphoton microscopy enables the detailed study of dynamic cellular processes in the kidney in living animals.
- Multiple recent technical advances are combining to greatly increase the capabilities of intravital microscopy and image analysis.
- Recent studies have utilized intravital microscopy to make important new insights into physiological and pathophysiological processes in the kidney, such as renal albumin handling and the pathogenesis of acute kidney injury in sepsis.
- Intravital microscopy is likely to play an increasingly important role in translational kidney research in the future.

defining the early part of the 21st century. Although their collective impact on intravital kidney imaging is yet to be fully realized, some important recent publications already provide clear pointers of what will become possible in the near future.

Long wavelength excitation

Arguably, the biggest practical constraint in intravital kidney imaging to date – at least in comparison with organs such as the brain – has been the limited depth of imaging possible. Typically, high-resolution images can only be acquired down to about 50 μm below the capsule [6], limiting the working area to the very outer cortex. Crucially, this means that important structures like glomeruli and early tubular segments cannot be routinely visualized, except in certain inbred strains of rat (which are prone to developing proteinuria and hypertension). The reason(s) why the kidney is so optically dense is(are) not fully understood, but probably relate to issues such as light scattering and optical aberrations (perhaps due to high blood flow) and a high background level of autofluorescence. The scattering of light in tissues is wavelength-dependent, and we have recently shown that by increasing the excitation wavelength (up to 1300 nm), it is possible to image significantly further into the kidney and visualize deeper glomeruli (Fig. 1) [7▪▪]. Moreover, autofluorescence is markedly lower at higher excitation wavelengths, meaning that when working with increasingly available far-red fluorescent probes, a much higher signal-to-background ratio can be achieved, which is important for high-resolution imaging of small structures. Other advantages of this approach include the possibility to image up to four different fluorescent probes with clear spectral separation (i.e. blue/green/red/far red) (Fig. 2) and also to elicit second harmonic-generated (SHG) and third harmonic-generated signals from important biological structures, like collagen and

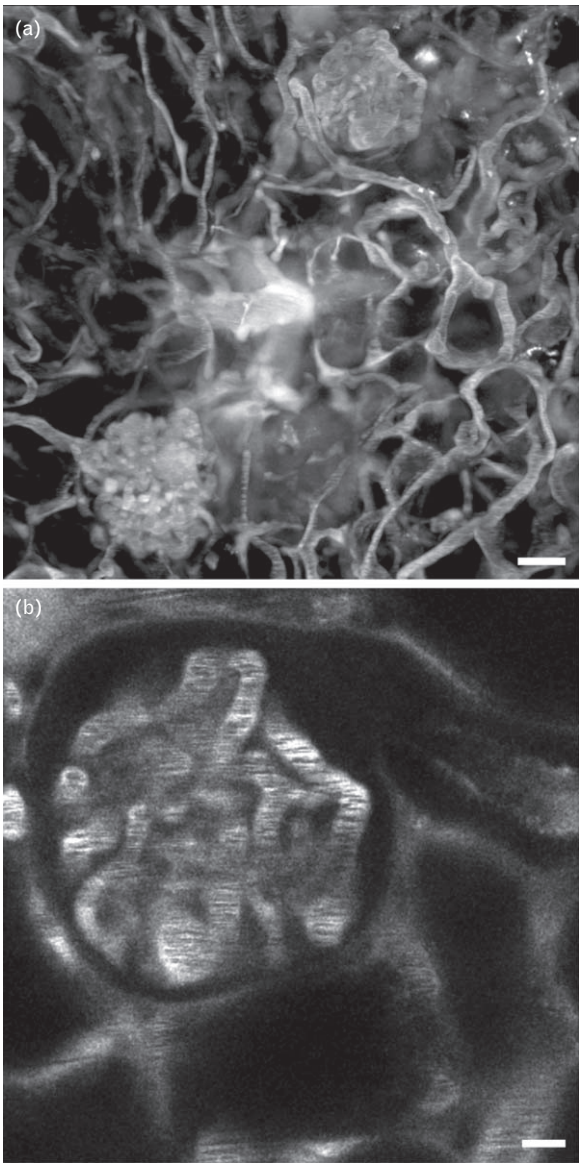

FIGURE 1. Intravital multiphoton kidney imaging with long wavelength excitation. The usage of long wavelength excitation and far-red probes allows deeper imaging in the mouse kidney than previously possible and the visualization of glomeruli. (a) Example of a three-dimensional rendered image acquired from a mouse injected with Alexa Fluor 647-labelled albumin excited at 1180 nm, showing two entire glomeruli and surrounding peritubular capillaries. The total stack was constructed from 125 individual two-dimensional images acquired at 1 μm *z* intervals. Scale bar = 30 μm. (b) A single plane is depicted from the above stack at a depth of 65 μm showing detailed morphology of glomerular capillaries. Scale bar = 10 μm.

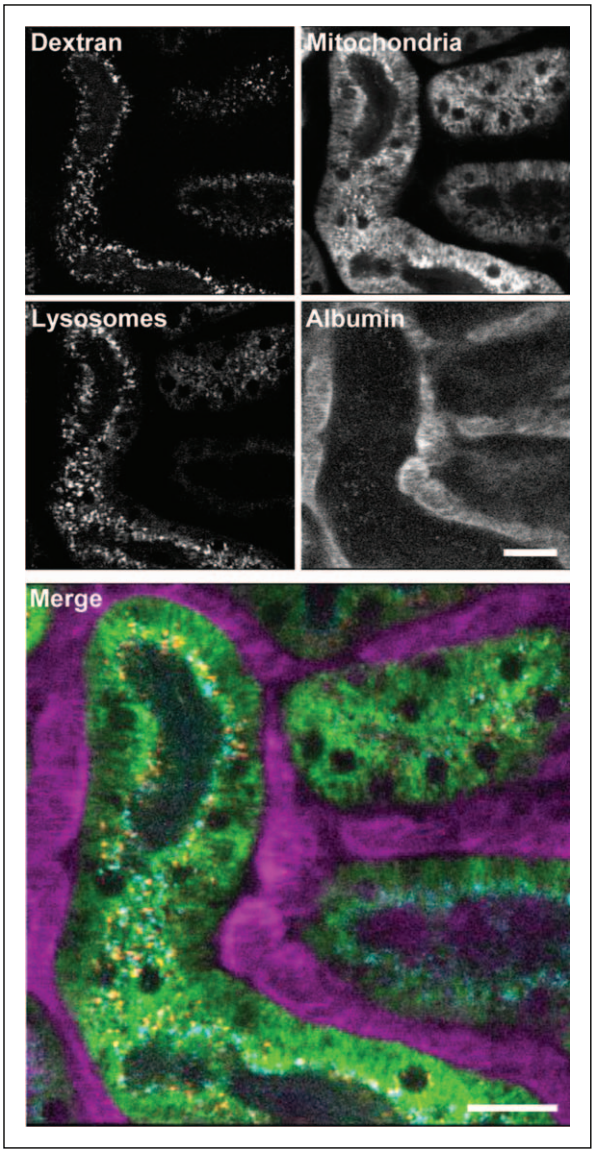

FIGURE 2. Simultaneous imaging of four different signals in the kidney. The broad-spectrum multiphoton excitation range of many fluorescent molecules and the addition of a far-red channel enables simultaneous imaging of multiple different probes with clear signal separation. In the example depicted, a mouse was injected with Cascade Blue Dextran (blue, a marker of endocytosis in the proximal tubule), Rhodamine 123 (green, a mitochondrial marker), Lysotracker (red, a lysosomal marker) and Alexa Fluor 647-labelled albumin (purple, a vascular marker). Scale bar = 10 μm.

lipids, respectively [8]. Meanwhile, advances in image analysis software, including image deconvolution and rendering, mean that serial two-dimensional images taken through the kidney can be used to reconstruct detailed three-dimensional architecture in living tissue (Fig. 1).

Adaptive optics

Although longer wavelength excitation clearly increases the depth of imaging possible in the kidney, it still remains far short of what can be achieved in other less optically dense organs, and there is a clear need for new and innovative solutions to this problem. Adaptive optics is a method that attempts to correct for aberrations produced by differences in the optical density in a specimen by measuring distortions in a wavefront, which can then be compensated for. It is widely used in astronomy, and its potential application to intravital imaging is increasingly being explored. However, compensating for aberrations in intact functioning organs, within which irregular dynamic processes like blood flow are occurring, represents a huge technical challenge. Nevertheless, a recent publication has suggested that the usage of adaptive optics can increase the effective imaging depth in the mouse brain to around 700 μm [9].

Transgenic animals expressing fluorescent proteins

To harness the full potential of intravital imaging for preclinical research, advances in hardware and software need to be matched by improvements in animal models. In the last few years, there has been an explosion in the number of new fluorescent sensors available to study key cellular processes; however, the usage of most of these is effectively confined at present to in-vitro experiments. In contrast, the range of signals that can currently be imaged in the kidney *in vivo* is far more limited. A major reason for this is that strategies used routinely in other organs to genetically express fluorescent sensors, such as bulk injection of viral vectors, have not proven successful in the kidney (although a recent study has suggested that injection via the renal vein could yield better results [10]). A more promising strategy is to genetically modify animals so that they express stable or inducible fluorescent reporters in cells of interest. This approach has been used recently to perform the very first intravital studies of Ca^{2+} signals in the nephron [11,12▪▪], which revealed several important new findings, including the fact that intracellular Ca^{2+} increases in podocytes in glomerular disease models.

Although some physiological events, such as Ca^{2+} signals, occur within seconds, other important processes take days or even weeks, timescales that are beyond the typical intravital imaging window of several hours. Repetitive imaging experiments are routinely performed in the brain by using an optical window, placed in an area of removed skull. Similar attempts to create windows in the abdomen have

been attempted [13], but are not as yet widely used. However, a recent study has shown that repetitive kidney imaging can be performed by simply replacing the kidney back within the abdominal compartment after each experiment [14]. When combined with podocin-confetti mice (which express either Cyan fluorescent protein (CFP), green fluorescent protein (GFP), yellow fluorescent protein (YFP), or red fluorescent protein (RFP) in individual podocytes), this approach allowed serial tracking of the same cells over several days and revealed the important finding that podocytes appear to migrate into the parietal layer in disease models. A study from another group also using podocyte-confetti mice has confirmed that podocytes appear relatively stationary under normal conditions, but show dramatic dynamic changes in disease models, including retraction of foot processes, detachment from the glomerulus and crawling along the tubular epithelium [15▪▪].

Traditionally, the generation of new genetically modified mouse lines has required significant investment of resources, but this is likely to become considerably quicker and easier in the future due to developments in genetic engineering. For example, the generation of knockout disease models has been greatly simplified by the recent discovery of the Clustered regularly interspaced short palindromic repeats Cas9 (CRISPR/Cas9) system. Genetic expression of fluorescent proteins is already routinely performed in zebrafish, which are ideally suited to intravital imaging due to their relative transparency. Such approaches have been utilized previously to perform studies of podocyte dynamics [16] and injury in disease models [17]. However, although there are obvious experimental advantages to the zebrafish model, these have to be weighed against the physiological distance between fish and human nephrons, and hence the translational relevance of data obtained.

Microendoscopy

Intravital imaging with a conventional MPM set-up is currently an invasive procedure and certainly not yet applicable to humans. However, the development of endoscopic microscopes offers the possibility to perform intravital imaging in a minimally invasive way. There is currently much interest in the potential usage of such instruments to perform 'virtual biopsies' in humans, whereby the histological nature of suspicious lesions could be assessed *in situ* [18]. It has recently been demonstrated that endoscopic microscopy can also be used to image the kidney in living anaesthetized mice [19], and although the quality of images that can be acquired is not as high as conventional MPM, this approach could clearly be advantageous for repetitive imaging studies in the same animal, perhaps even when awake.

Fluorescence lifetime imaging

Many fluorophores have overlapping emission spectra, which can lead to bleed through of signals and make clear spectral separation challenging. One possible solution to this problem is to measure the time decay of fluorescence (the lifetime), which is unique to each fluorophore and its microenvironment. Fluorescence lifetime imaging (FLIM) with MPM has been established for some time and can be used to distinguish important endogenous fluorophores, like Nicotinamide adenine dinucleotide (NADH) and NADPH, which have virtually identical excitation/emission spectra, but very different physiological roles. Moreover, as the lifetime of these molecules changes when they are bound or free, FLIM can be used to make measurements of cellular metabolism [20]. Although FLIM has been used in intravital studies of organs like the skin [21] and liver [22], it is yet (to our knowledge) to be applied to the kidney. The prerequisite for both a very high level of tissue stability and sufficiently bright signal, to accurately measure fluorescence decay over time, could make FLIM challenging in the kidney in living animals. However, if these technical hurdles can be overcome, FLIM could in principle offer some clear advantages, given the high level of endogenous autofluorescence in the kidney. In the meantime, a recent study using fixed specimens from a ureteric ligation model of chronic kidney disease has shown that FLIM can be used to separate autofluorescence and SHG signals [23▪]. As the latter arise from collagen, this allows quantification of the extent of fibrosis within the specimen, without the need for any special stains.

Tissue clearing techniques

Although not strictly speaking an intravital technique, there is nevertheless huge interest currently in the application of tissue clearing techniques to whole organ imaging. The basic principle of this approach is to render fixed tissues transparent by removing lipid components and by increasing the refractive index of the solvent, whilst retaining proteins and fluorescent markers. The breakthrough demonstration of this technique was in the brain, using the so-called CLARITY protocol [24], and numerous examples have followed since. A recent study from Klingberg *et al.* [25▪] has nicely shown the potential of tissue clearing for kidney imaging. They used fluorescently labelled antibodies to stain

capillaries in the mouse kidney, which enabled visualization of glomeruli. Using tissue clearing, they were then able to image through the entire organ. By using a particular type of fluorescence imaging particularly suited to rapid three-dimensional imaging (called light-sheet microscopy), and automated image processing, they could quantify the number and volume of glomeruli in the whole kidney. As antibody staining of proteins of interest remains a backbone of renal research, tissue clearing techniques could clearly have an important future role in enabling routine three-dimensional analysis of expression patterns. However, effective labelling of antigens in an intact organ and preservation of binding during the clearing process still remain significant technical barriers in many cases, and further methodological refinements are required in this field.

NEW FRONTIERS IN UNDERSTANDING OF KIDNEY (PATHO)PHYSIOLOGY

As the technical frontiers of intravital imaging march forward ever more quickly, so too inevitably will the limits of understanding of whole organ physiology. Intravital microscopy has provided numerous fascinating new insights in translational kidney research in the last decade [3]. In the following sections, we will highlight two particularly important subject areas in which imaging has recently made a real impact.

Renal handling of albumin

The hallmark of many kidney diseases is an increase in urinary excretion of albumin, which is widely associated with both accelerated decline of renal function and increased risk of cardiovascular disease [26]. Moreover, albuminuria is often used as a biomarker or surrogate endpoint in clinical studies. There is, therefore, much interest in studying how exactly the kidney handles albumin. The classical view – driven largely by older invasive micropuncture studies – is that only a relatively small amount of albumin is filtered, due to its size and negative charge [27]. However, more recent intravital imaging studies have challenged this paradigm, with the observation that fluorescently labelled albumin injected systemically rapidly appears in the apical part of cells in the proximal tubule, implying that tubular reuptake plays a far more important role than previously appreciated [28]. It has also been proposed, partly on the basis of imaging studies, that albumin may be trafficked directly across proximal tubule cells and back into the blood stream [29,30]. These surprising findings have huge implications for clinical practice and have inevitably generated much controversy; the picture is further complicated by the fact that different groups using the same imaging techniques have reported very different values for the glomerular sieving coefficient [31,32].

Resolving these major discrepancies will not be easy; as with most polarized debates, it seems likely that the truth lies somewhere in between the two extreme opposing views. In the meantime – assuming all can agree that at least some albumin is filtered – two new intravital imaging studies have provided important new insights into how this might be regulated. First, Schiessl *et al.* [33▪▪] have shown that in response to angiotensin II infusion, podocytes can perform megalin-mediated endocytosis of fluorescently labelled albumin, which is subsequently trafficked across the cell and released into the urinary space. Interestingly, uptake could be blocked by an angiotensin II receptor inhibitor, which could provide a new explanation for an old observation that these drugs decrease proteinuria in various glomerular diseases. Meanwhile, Wagner *et al.* [34▪▪] have recently suggested that proximal tubule uptake of albumin might be regulated, as part of a homeostatic mechanism to maintain the plasma concentration in the face of changes in albumin intake or excretion. In support of this notion, they found that proximal tubule uptake decreased when animals were injected with exogenous albumin (a model of systemic albumin excess), but that it increased in response to podocyte damage and increased glomerular sieving (a model of systemic albumin depletion). Although these findings are highly intriguing, the molecular mechanism(s) that could underlie such a regulatory process remain to be elucidated.

Sepsis and acute kidney injury

Sepsis is a major cause of acute kidney injury (AKI) and is associated with significant morbidity and mortality [35]. Unfortunately, our basic understanding of the pathogenesis of septic AKI remains far from complete, and treatment options are accordingly limited. AKI in sepsis is likely to be a complex and multifactorial process, involving dynamic interplay between the microvasculature, kidney tubules and both resident and circulating immune cells [36]. Or to put it another way, a perfect disease to study with live imaging techniques.

Three fundamental and unresolved questions continue to dominate the sepsis-AKI field. First, does tubular damage occur directly (e.g. due to endotoxin) or indirectly (e.g. due to reduced oxygen delivery)? Second, why do patients develop oliguria

even in the absence of clear reductions in renal blood flow? Third, is the resulting immune response protective or harmful? Several groups have recently utilized intravital microscopy to try to answer these questions. First, Dagher *et al*. have shown that fluorescently labelled endotoxin [lipopolysaccharide (LPS)] is filtered by the kidney and taken up in early (S1) proximal tubule segments [37]. However, rather than directly causing damage in this segment, evidence of oxidative stress was surprisingly observed downstream in later S2 segments, perhaps due to some kind of crosstalk process, involving paracellular communication. Meanwhile, another group have shown that LPS causes a reduction in tubular flow rate, in the absence of a fall in either blood pressure or glomerular filtration rate, which can only be partially overcome by supplementation with intravenous fluids [38▪▪]. Decreased tubular flow could be explained, at least in part, due to proximal tubule cell swelling and partial luminal obstruction. Finally, two recent studies have looked at the role of immune cells. In a follow-up study to the one previously mentioned, Dagher *et al*. showed that preconditioning with small doses of endotoxin protects against subsequent oxidative stress in proximal tubules and that this effect requires macrophages, which increase in number in the kidney during sepsis and cluster around proximal tubules [39▪▪]. Using a caecal ligation and puncture model, Chousterman *et al*. also showed that circulating monocytes infiltrate the kidney and appear to have a protective effect, adhering to the vasculature in a process that is dependent on the chemokine receptor CX3CR1 [40▪▪].

Taken together, these findings suggest that AKI in sepsis does involve direct tubular toxicity, which could in part explain reductions in urine flow, and that immune cells can have a protective role in response to injury. Further work will be required to definitively confirm or refute these tentative conclusions, but in the meantime these studies nicely illustrate the power of intravital microscopy to help elucidate complex pathological processes in real time, involving multiple different interacting cell types.

CONCLUSION AND FUTURE DIRECTIONS

With a few notable exceptions, it could be argued that translational kidney research is failing in general, especially in diseases such as AKI, with negative clinical trials currently greatly outnumbering the positive [41]. Concurrent with this – and surely not coincidental – is the perception of a relative decline in the standing of basic renal research [42]. To rectify this situation, we need a vastly better fundamental understanding of how the kidney works, at both a cellular and whole organ level, and what exactly changes (and when) in disease states. To achieve this, we need to move away from unrepresentative in-vitro models, small datasets and unsophisticated experimental techniques, and instead embrace the opportunities offered by the current whirlwind technological revolution to study integrative systems biology (or physiology, as it was once known) in animal models that can realistically reflect the multilayer complexity of human diseases. This will inevitably entail a rather more honest admission of the ongoing pivotal role of animal experiments in translational research. It is now demonstrably possible, using intravital microscopy, to make detailed quantitative measurements of complex and dynamic cellular processes in the functioning kidney and to assess how they are altered in real time in pathological states or in response to putative treatments. Moreover, rapid developments in multiple different, but complementary, fields will all combine to move the frontiers forward even faster in the future, leading to applications that are yet to be even imagined. This will bring with it new challenges, such as how to effectively store and smartly analyse the vast amount of data that will be generated. Nevertheless, these are evidently exciting times for biomedical imaging, and it seems increasingly likely that we will soon have the tools to address many intriguing and important unanswered questions in kidney research. We just have to use them.

Acknowledgements

A.M.H., C.D.S. and D.H. are supported by The Swiss National Centre for Competence in Research (NCCR) Kidney Control of Homeostasis, The Swiss National Science Foundation, The Clinical Research Priority Program 'Molecular Imaging Network Zurich' and The Zurich Centre for Integrative Human Physiology. They also gratefully acknowledge the assistance of the University of Zurich Centre for Microscopy and Image Analysis (ZMB).

Financial support and sponsorship

None.

Conflicts of interest

There are no conflicts of interest.

REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:
- ▪ of special interest
- ▪▪ of outstanding interest

1. Helmchen F, Denk W. Deep tissue two-photon microscopy. Nat Methods 2005; 2:932–940.

2. Ashworth SL, Sandoval RM, Tanner GA, Molitoris BA. Two-photon microscopy: visualization of kidney dynamics. Kidney Int 2007; 72:416–421.
3. Peti-Peterdi J, Burford JL, Hackl MJ. The first decade of using multiphoton microscopy for high-power kidney imaging. Am J Physiol Renal Physiol 2012; 302:F227–F233.
4. Peti-Peterdi J, Kidokoro K, Riquier-Brison A. Novel in vivo techniques to visualize kidney anatomy and function. Kidney Int 2015; 88:44–51.
5. Schiessl IM, Castrop H. Deep insights: intravital imaging with two-photon microscopy. Pflugers Arch 2016; 468:1505–1516.
6. Schiessl IM, Bardehle S, Castrop H. Superficial nephrons in BALB/c and C57BL/6 mice facilitate in vivo multiphoton microscopy of the kidney. PLoS One 2013; 8:e52499.
7. ■■ Schuh CD, Haenni D, Craigie E, *et al.* Long wavelength multiphoton excitation is advantageous for intravital kidney imaging. Kidney Int 2016; 89:712–719.

This study showed that the usage of long wavelength excitation light and far-red fluorophores allows deeper imaging in the intact kidney.

8. Debarre D, Supatto W, Pena AM, *et al.* Imaging lipid bodies in cells and tissues using third-harmonic generation microscopy. Nat Methods 2006; 3:47–53.
9. Wang K, Sun W, Richie CT, *et al.* Direct wavefront sensing for high-resolution in vivo imaging in scattering tissue. Nat Commun 2015; 6:7276.
10. Corridon PR, Rhodes GJ, Leonard EC, *et al.* A method to facilitate and monitor expression of exogenous genes in the rat kidney using plasmid and viral vectors. Am J Physiol Renal Physiol 2013; 304:F1217–F1229.
11. Burford JL, Villanueva K, Lam L, *et al.* Intravital imaging of podocyte calcium in glomerular injury and disease. J Clin Invest 2014; 124:2050–2058.
12. ■■ Szebenyi K, Furedi A, Kolacsek O, *et al.* Visualization of calcium dynamics in kidney proximal tubules. J Am Soc Nephrol 2015; 26:2731–2740.

This study used genetic expression of a calcium-sensitive fluorescent probe to provide the first detailed description of intracellular calcium signals in kidney tubules *in vivo*.

13. Ritsma L, Steller EJ, Ellenbroek SI, *et al.* Surgical implantation of an abdominal imaging window for intravital microscopy. Nat Protoc 2013; 8:583–594.
14. Hackl MJ, Burford JL, Villanueva K, *et al.* Tracking the fate of glomerular epithelial cells in vivo using serial multiphoton imaging in new mouse models with fluorescent lineage tags. Nat Med 2013; 19:1661–1666.
15. ■■ Brahler S, Yu H, Suleiman H, *et al.* Intravital and kidney slice imaging of podocyte membrane dynamics. J Am Soc Nephrol 2016; 27:3285–3290.

This study used genetically modified 'confetti mice' to provide further evidence that podocytes are relatively static at rest, but become highly dynamic under pathological conditions.

16. Endlich N, Simon O, Gopferich A, *et al.* Two-photon microscopy reveals stationary podocytes in living zebrafish larvae. J Am Soc Nephrol 2014; 25:681–686.
17. Zhou W, Hildebrandt F. Inducible podocyte injury and proteinuria in transgenic zebrafish. J Am Soc Nephrol 2012; 23:1039–1047.
18. Cicchi R, Sturiale A, Nesi G, *et al.* Multiphoton morpho-functional imaging of healthy colon mucosa, adenomatous polyp and adenocarcinoma. Biomed Opt Express 2013; 4:1204–1213.
19. Ducourthial G, Leclerc P, Mansuryan T, *et al.* Development of a real-time flexible multiphoton microendoscope for label-free imaging in a live animal. Sci Rep 2015; 5:18303.
20. Blacker TS, Mann ZF, Gale JE, *et al.* Separating NADH and NADPH fluorescence in live cells and tissues using FLIM. Nat Commun 2014; 5:3936.
21. Seidenari S, Arginelli F, Bassoli S, *et al.* Multiphoton laser microscopy and fluorescence lifetime imaging for the evaluation of the skin. Dermatol Res Pract 2012; 2012:810749.
22. Thorling CA, Liu X, Burczynski FJ, *et al.* Multiphoton microscopy can visualize zonal damage and decreased cellular metabolic activity in hepatic ischemia-reperfusion injury in rats. J Biomed Opt 2011; 16:116011.
23. ■ Ranjit S, Dobrinskikh E, Montford J, *et al.* Label-free fluorescence lifetime and second harmonic generation imaging microscopy improves quantification of experimental renal fibrosis. Kidney Int 2016; 90:1123–1128.

This study demonstrated that the measurement of second harmonic signals generated by collagen can be used to quantify the extent of fibrosis in fixed kidney specimens acquired from animal models of chronic kidney disease.

24. Chung K, Wallace J, Kim SY, *et al.* Structural and molecular interrogation of intact biological systems. Nature 2013; 497:332–337.
25. ■ Klingberg A, Hasenberg A, Ludwig-Portugall I, *et al.* Fully automated evaluation of total glomerular number and capillary tuft size in nephritic kidneys using lightsheet microscopy. J Am Soc Nephrol 2017; 28:452–459.

This study demonstrated that tissue clearing techniques and light-sheet microscopy can be combined to rapidly image whole fixed kidneys.

26. Matsushita K, vand V, Astor BC, *et al.* Association of estimated glomerular filtration rate and albuminuria with all-cause and cardiovascular mortality in general population cohorts: a collaborative meta-analysis. Lancet 2010; 375:2073–2081.
27. Christensen EI, Birn H, Rippe B, Maunsbach AB. Controversies in nephrology: renal albumin handling, facts, and artifacts! Kidney Int 2007; 72:1192–1194.
28. Russo LM, Sandoval RM, McKee M, *et al.* The normal kidney filters nephrotic levels of albumin retrieved by proximal tubule cells: retrieval is disrupted in nephrotic states. Kidney Int 2007; 71:504–513.
29. Tenten V, Menzel S, Kunter U, *et al.* Albumin is recycled from the primary urine by tubular transcytosis. J Am Soc Nephrol 2013; 24:1966–1980.
30. Dickson LE, Wagner MC, Sandoval RM, Molitoris BA. The proximal tubule and albuminuria: really! J Am Soc Nephrol 2014; 25:443–453.
31. Tanner GA. Glomerular sieving coefficient of serum albumin in the rat: a two-photon microscopy study. Am J Physiol Renal Physiol 2009; 296:F1258–F1265.
32. Sandoval RM, Wagner MC, Patel M, *et al.* Multiple factors influence glomerular albumin permeability in rats. J Am Soc Nephrol 2012; 23:447–457.
33. ■■ Schiessl IM, Hammer A, Kattler V, *et al.* Intravital imaging reveals angiotensin II-induced transcytosis of albumin by podocytes. J Am Soc Nephrol 2016; 27:731–744.

This study used intravital microscopy to provide evidence that podocytes can transport albumin and that this process is regulated by angiotensin II.

34. ■■ Wagner MC, Campos-Bilderback SB, Chowdhury M, *et al.* Proximal tubules have the capacity to regulate uptake of albumin. J Am Soc Nephrol 2016; 27:482–494.

This study used intravital imaging to show that the amount of albumin taken up by the proximal tubule via receptor-mediated endocytosis changes in response to systemic albumin overload or depletion.

35. Alobaidi R, Basu RK, Goldstein SL, Bagshaw SM. Sepsis-associated acute kidney injury. Semin Nephrol 2015; 35:2–11.
36. Gomez H, Ince C, De Backer B, *et al.* A unified theory of sepsis-induced acute kidney injury: inflammation, microcirculatory dysfunction, bioenergetics, and the tubular cell adaptation to injury. Shock 2014; 41:3–11.
37. Kalakeche R, Hato T, Rhodes G, *et al.* Endotoxin uptake by S1 proximal tubular segment causes oxidative stress in the downstream S2 segment. J Am Soc Nephrol 2011; 22:1505–1516.
38. ■■ Nakano D, Doi K, Kitamura H, *et al.* Reduction of tubular flow rate as a mechanism of oliguria in the early phase of endotoxemia revealed by intravital imaging. J Am Soc Nephrol 2015; 26:3035–3044.

Using intravital microscopy and an endotoxemia model of septic acute kidney injury (AKI), this study showed that in the absence of a decrease in blood pressure, oliguria can be explained by decreases in tubular flow rate due to proximal tubular cell damage and swelling.

39. ■■ Hato T, Winfree S, Kalakeche R, *et al.* The macrophage mediates the renoprotective effects of endotoxin preconditioning. J Am Soc Nephrol 2015; 26:1347–1362.

Building on the previous novel finding that endotoxin induces oxidative stress in the S2 segment of proximal tubules, this study showed that the protective effect of prior low-dose injections of endotoxin (so-called preconditioning) requires the presence of macrophages.

40. ■■ Chousterman BG, Boissonnas A, Poupel L, *et al.* Ly6 chigh monocytes protect against kidney damage during sepsis via a CX3CR1-dependent adhesion mechanism. J Am Soc Nephrol 2016; 27:792–803.

This study provided evidence that monocytes infiltrate the kidney during septic AKI and have protective effects.

41. de Caestecker CM, Humphreys BD, Liu KD, *et al.* Bridging translation by improving preclinical study design in AKI. J Am Soc Nephrol 2015; 26:2905–2916.
42. Al-Awqati Q. Basic research in nephrology: are we in decline? J Am Soc Nephrol 2012; 23:1611–1616.